PCR-based coprodiagnostic tools reveal dogs as reservoirs of zoonotic ancylostomiasis caused by *Ancylostoma ceylanicum* in temple communities in Bangkok

Rebecca J. Traub a,*, Tawin Inpankaew b, Chantira Sutthikornchai c, Yaowalark Sukthana c, R.C. Andrew Thompson d

a School of Veterinary Science, University of Queensland, St Lucia, Queensland 4072, Australia
b Department of Parasitology, Faculty of Veterinary Medicine, Kasetsart University, Bangkok, Thailand
c Department of Protozoology, Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand
d WHO Collaborating Centre for the Molecular Epidemiology of Parasitic Infections, School of Veterinary and Biomedical Sciences and the State Agricultural Biotechnology Centre, Murdoch University, Murdoch, Western Australia 6150, Australia

Received 15 January 2008; received in revised form 7 April 2008; accepted 5 May 2008

Abstract

A survey of gastrointestinal parasites of dogs and humans from temple communities in Bangkok revealed that 58% of dogs and 3.4% of humans, among those sampled, were infected with hookworms utilising faecal flotation techniques and microscopy. A previously established polymerase chain reaction (PCR)-RFLP approach was utilised to determine the species of hookworms infecting dogs found positive for hookworm eggs. Single infections with *Ancylostoma ceylanicum* and *Ancylostoma caninum* were recorded in 77% and 9% of hookworm positive dogs, respectively and mixed infections with both species of *Ancylostoma* were recorded in 14% of dogs. A single-step PCR for the multiplex detection of *Ancylostoma* species and *Necator americanus* DNA in human faeces was developed and applied to characterise the species of hookworms in microscopy positive individuals. Single infection with *N. americanus* was recorded in five and *A. ceylanicum* infection in two, out of seven individuals positive for hookworm. This study demonstrates that humans are at risk of acquiring infection with *A. ceylanicum* in communities where this species of hookworm is endemic in dogs.

© 2008 Elsevier B.V. All rights reserved.

Keywords: *Ancylostoma*; Hookworms; Dogs; Zoonoses; Thailand; PCR

1. Introduction

The hookworm species *Ancylostoma caninum*, *Ancylostoma braziliense* and *Ancylostoma ceylanicum* are known to be endemic in dogs (and cats) in India (Traub et al., 2007, 2004) and south east Asia (Choo et al., 2000; Setasuban et al., 1976; Yoshida et al., 1968, 1973). Canine hookworms are important not only from a veterinary stand-point but also a public health perspective, as all hookworm species of dogs are potentially zoonotic and capable of producing a degree of skin irritation referred to as “creeping eruptions” or cutaneous larva migrans (CLM) in humans (Mapleston, 1933; Chaudhry and Longworth, 1989; Carroll and Grove, 1986; Landmann and Prociv, 2003). *A. braziliense* is the most frequently implicated aetiological agent (Chaudhry and Longworth, 1989; Malgor et al., 1996) and is the species responsible for prolonged
CLM, with tracts in the skin being recorded for over 100 days in some cases (Dove, 1932). CLM is commonly reported in travellers returning from the tropics (Bouchaud et al., 2001; Jelinek et al., 1994; Tremblay et al., 2000) and in areas endemic for canine hookworm infection (Malgor et al., 1996; Taranto et al., 2000). In addition, the recognition that *A. caninum* may occasionally grow to immature adults in the small intestine of humans leading to eosinophilic enteritis has resulted in cases reports from Queensland (Croese et al., 1994b; Loukas et al., 1992), the Philippines, South America, Israel (Croese et al., 1994a), the US (Khoshoo et al., 1995, 1994) and Egypt (Bahgat et al., 1999). However, *A. ceylanicum* is the only species of canine hookworm known to produce patent infections in humans. This has been demonstrated both experimentally (Carroll and Grove, 1986; Wijers and Smit, 1966) and naturally. The last detailed reports of natural infections with *A. ceylanicum* in humans were published over 40 years ago, between the mid 1960s to early 1970s (Anten and Zuidema, 1964; Areekul et al., 1970; Chowdhury and Schad, 1972; Velasquez and Cabrera, 1968; Yoshida et al., 1968). Since then, although *A. ceylanicum* is known to be an endemic and widely distributed hookworm in dogs and cats in Asia, and an emerging zoonosis in Australia (Palmer et al., 2007), the parasite has been regarded as a ‘rare’ and ‘abnormal’ hookworm of humans and largely overlooked in human parasite surveys.

A recent simultaneous survey of gastrointestinal parasites in dogs and humans residing at temple communities in Bangkok, Thailand found hookworms to be the most common helminth recovered with a prevalence of 58.1% (95% CI: 51.7–64.6) in dogs and 3.4% (95% CI: 0.9–5.9%) in humans (Inpankaew et al., 2007). Thai temples/monasteries and their surrounding communities consist of monks, nuns and families from low socioeconomic backgrounds. Companion animal ownership is a very popular practice in Bangkok, however due to religious and cultural beliefs, euthanasia of sick or unwanted animals is strongly discouraged and therefore animals are commonly abandoned at the temple grounds where monks are obliged to feed and care for them. An estimated 500 temples lie in Bangkok city alone, with approximately 20,000 semi-domesticated dogs and 30,000 monks residing within them in sometimes unhygienic, overcrowded living conditions that are conducive for the transmission of zoonotic parasites. The primary aim of the current study was to determine the role of dogs as zoonotic reservoirs for *A. ceylanicum* in temple communities in Bangkok by using a combination of molecular diagnostic and epidemiological tools.

Recently, Palmer et al. (2007) adapted a polymerase chain reaction (PCR)-RFLP originally developed by Traub et al. (2004) to differentiate canine (and feline) *Ancylostoma* species directly from faeces found positive for hookworm eggs by flotation techniques. Palmer et al. (2007) applied this PCR-RFLP to a large scaled epidemiological survey of canine and feline hookworm species in Australia (Palmer et al., 2007). The application of this molecular tool obviated the need for tedious morphological identification of adult hookworms following treatment with anthelmintics or at post-mortem and was utilised to differentiate the species of hookworms in microscopy positive dogs. A separate PCR capable of amplifying both *Necator americanus* and *Ancylostoma* species (*Ancylostoma duodenale* and *A. ceylanicum*) in a multiplex, single-step reaction was developed and utilised to characterise the species of hookworms in microscopy positive humans.

2. Materials and methods

2.1. Collection of faecal samples and interviews

Single faecal samples were randomly collected from of a total of 204 humans and 229 dogs from 20 temples and their surrounding communities in Bangkok city between the months of June to September in 2004 and in 2005. Temples were selected on the basis of convenience (within a 50 km radius of the Faculty of Tropical Medicine, Mahidol University). Specific data were collected from each individual human participant as well as their dog(s) with regards to risk factors for parasitic infection. Data were collected on the dog’s age, breed, gender, diet, defaecation and roaming patterns, frequency of deworming, vaccination status and access to a veterinarian. Specific data were collected from each individual human participant on their socioeconomic status, gender, age, educational background, diet, occupation, defaecation practices, type of water drunk and treatment of water prior to consumption, utilisation of footwear when outdoors, household crowding and dog ownership/contact. Individuals were also asked to state if they were suffering from clinical signs such as abdominal pain, dyspepsia, diarrhoea, weakness, anorexia and weight loss and asked to classify their health status as ‘excellent’, ‘average’ or ‘poor’. Interviewer bias was kept to a minimum by having a questionnaire with ordered and specific questions and procedures to follow. Faeces were collected from the rectum of the dogs by qualified veterinarians and veterinary assistants. Humans were
handed faecal pots with their names on it for collection the following day. Verbal consent was obtained from each human participant or their parent/guardian prior to participation. The study was approved by the Murdoch University Human and Animal Ethics Committees of Western Australia.

2.2. Parasitological procedure

Fresh faecal samples were transported back to the Faculty of Protozoology, Mahidol University, Bangkok and refrigerated until screened using zinc sulphate and sodium nitrate flotation within 24 h of collection. The remainder of the faecal sample was stored in 20% dimethylsulphoxide (DMSO) saturated with salt for transport to Murdoch University, Western Australia for further molecular testing.

2.3. Molecular procedures—DNA extraction

Table 1 lists the species and sources of the adult hookworms utilised as positive controls in this study. DNA was extracted from adult worms using the Qiagen DNeasy Blood and Tissue Kit according to manufacturer’s instructions. Dog and human faecal samples microscopically positive for strongyle eggs were subjected to DNA extraction and molecular characterisation. Two hundred milligrams of faeces were subjected to DNA extraction and molecular characterisation. Two hundred milligrams of faeces were suspended in 1.4 ml ATL tissue lysis buffer (Qiagen, Hilden, Germany) and this suspension subjected to 3–5 cycles of freezing at liquid nitrogen temperatures followed by thawing at 96–98 °C. DNA was then isolated from the supernatant using the QIAamp DNA Mini Stool Kit according to manufacturer’s instructions. Final elutions of DNA were made in 50 μl of elution buffer instead of 200 μl as recommended by the manufacturer.

2.4. Molecular methods—PCR-RFLP of canine hookworms

PCR-RFLP characterisation of canine hookworms was performed according to Palmer et al. (2007). RTGHF1 (5'-CGTGCTAGTCTCTCAGGACTTGG-3') and RTABCR1 (5'-CGGGAATTGCTATAAGCAAGTGC-3') were used to amplify a 545 bp region of the internal transcribed spacer (ITS)-1, 5.8S and ITS-2 of A. caninum, A. ceylanicum and Uncinaria stenocephala. In a separate PCR a 673 bp region of the ITS-1, 5.8S and ITS-2 of A. braziliense was amplified using RTGHF1 and the highly specific reverse primer RTAYR1 (5'-CTGCTGAAAAGTCTCAAGTCC-3'). Amplified ITS PCR products of RTGHF1-RTABCR1 were subjected to direct digestion with HinF1in order to differentiate A. caninum from A. ceylanicum and U. stenocephala. Restriction enzyme RSaI was then used when necessary, to differentiate U. stenocephala from A. ceylanicum. The RFLP reaction for HinFI (Promega) and RSaI (Promega) restriction endonuclease were identical. Ten microlitres of PCR product were digested with 0.5 μl (5 units) of a restriction endonuclease at 37 °C for 16 h in a volume of 20 μl. Positive controls of A. caninum, A. ceylanicum, A. braziliense and U. stenocephala listed in Table 1 were utilised to validate the PCRs and PCR-RFLPs.

2.5. Molecular methods—PCR and DNA sequencing of human hookworms

A forward primer RTHW1F (5'-GATGAGCATTGCWTAATGCGCG-3') and reverse primer RTHW1R (5'-GCAAGTCTCGTGCAACG-3') were designed and utilised to amplify an approximately 485 bp and 380 bp section of the ITS1, 5.8S and ITS2 regions of N. americanus and Ancylostoma spp. The PCR was carried out in a 25 μl volume with the final mix containing 2.5 μl of 10× PCR buffer, 2.5 μl of 25 mM MgCl2, 1.0 μl of 20 mM dNTPs, 12.5 pmol of each primer, 200 μm of each dNTP, 1 unit (0.2 μl) of Thal plus (Biotec International, Perth, Australia) and 1 μl of template DNA. Samples were heated to 94 °C for 2 min, 64 °C for 1 min, 72 °C for 2 min followed by 50 cycles of 94 °C for 30 s, 64 °C for 30 s, 72 °C for 30 s (extension), and a final extension at 72 °C for 7 min. Titration experiments were conducted to determine the analytical sensitivity of the PCR for the detection of N. americanus, A. duodenale and A. ceylanicum DNA. The assay’s ability to detect artificially mixed infections with varying ratios of N. americanus and A. ceylanicum was also assessed.

All PCR positive human hookworm products were subjected to DNA sequencing. PCR products were purified using an UltraClean™ GelSpin DNA Purification Kit (MO BIO Laboratories Inc., Solana Beach, CA, USA) according to the recommendations of the manufacturer and sequenced in both directions using the Big Dye Terminator system, Version 3.1 (Applied Biosystems, Foster City, CA) on an ABI 373 x 1 capillary sequencer. Sequence chromatograms were edited and analysed using the software program Finch TV Version 1.4.0 (©Geospiza Inc.). Human hookworm sequences were aligned and compared to previously published sequences of A. duodenale (GenBank accession numbers AJ001679, AJ001594), N. americanus
(AJ001680, Y11734, AF217891) and A. ceylanicum (DQ780009, DQ831517) and the sequences generated from positive controls of N. americanus, A. duodenale and A. ceylanicum using BioEdit (Hall, 1999).

2.6. Statistical analyses

Univariate associations between the prevalence of hookworms in dogs and humans and host, behavioural and environmental factors were initially made using Chi-square results for independence and ANOVA (continuous variables). Logistic multiple regression was used to quantify the association between the prevalence of hookworms using each test and each variable after adjusting for other variables. Only variables significant at $p \leq 0.25$ in the univariate analyses were considered eligible for inclusion in the logistic multiple regression (Frankena and Graat, 1997; Hosmer and Lemeshow, 1989). Backward elimination was used to determine which factors could be dropped from the multivariable model. The likelihood-ratio Chi-squared statistic was calculated to determine the significance at each step of the model building. The level of significance for a factor to remain in the final model was set at 10%. The goodness of fit of the model was assessed with the Hosmer–Lemeshow statistic (Lemeshow and Hosmer, 1982). Data were analysed and statistical comparisons were performed using SPSS (SPSS for Windows, Version 14.0, Rainbow Technologies) and Excel 2002 (Microsoft).

3. Results

Nearly all dogs (93%) defaecated outdoors and faeces were removed from the grounds approximately once every week by 80% of monks or owners, although this was not always observed by the interviewer. The majority of humans reported defaecating in indoor latrines (95.6%). Sixty percent of participants reported wearing footwear at all times when outdoors, 31% wore footwear occasionally and 9% rarely wore footwear.

The majority of the population dewormed themselves on an infrequent to rare basis, 11% of the population dewormed themselves on a regular basis (every 6 months to 1 year) and the remainder were unsure of their deworming status.

A total of 133 dogs (prevalence 58.1%) were microscopy positive for hookworm eggs. The PCR-RFLP was able to successfully amplify and characterise 91.7% of samples. Of these, single infections with A. ceylanicum and A. caninum were recorded in 77% and 9% of dogs, respectively and mixed infections with both species of Ancylostoma were recorded in 14% of dogs.

Dogs less than 1 year (OR 2.1, 95% CI: 1.0–4.1, $p = 0.04$), those living in the immediate surrounding of more than 10 dogs (OR 2.8, 95% CI: 1.0–7.4, $p = 0.04$), those that were not wormed at least once in the past 12 months (OR 7.2, 95% CI: 2.7–19.2, $p = 0.00$) and those that were entire (OR 3.1, 95% CI: 1.5–6.7, $p = 0.00$) were more likely to be infected with hookworms (Inpankaew et al., 2007). No risk factors or correlations were found for the prevalence of individual hookworm species harboured by dogs.

Using the primer pair RTHW1F and RTHW1R, DNA from adult N. americanus gave a specific 485 bp product and DNA from adults of A. duodenale and A. ceylanicum gave specific products of 380 bp. The lowest quantity of DNA that could be amplified from individual adults of N. americanus, A. duodenale and A. ceylanicum was estimated at 2.5 pg. Appropriate sized amplicons were produced in reactions artificially mixing DNA of N. americanus separately with A. duodenale and A. ceylanicum in ratios of 1:1, 1:2, 1:3, 3:1 and 2:1.

Out of the seven humans found microscopy positive for hookworms (prevalence 3.4%), the PCR was able to successfully amplify all seven samples, which were subsequently characterised. Single infection with N. americanus was recorded in five and A. ceylanicum infection in two out of seven hookworm positive individuals. The first individual positive for A. ceylanicum was a 60-year-old lady who complained

<table>
<thead>
<tr>
<th>Species</th>
<th>Source</th>
<th>Origin</th>
<th>Year specimen collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ancylostoma caninum</td>
<td>Dog, Bangkok, Thailand</td>
<td>–</td>
<td>2004</td>
</tr>
<tr>
<td>Ancylostoma braziliense</td>
<td>Cat, Cocos (Keeling) Islands, Australia</td>
<td>–</td>
<td>2005</td>
</tr>
<tr>
<td>Uncinaria stenocephala</td>
<td>Dog, Hannover, Germany</td>
<td>–</td>
<td>2004</td>
</tr>
<tr>
<td>Ancylostoma ceylanicum</td>
<td>Hamster*, Nottingham, U.K.</td>
<td>Dog, India</td>
<td>2006</td>
</tr>
<tr>
<td>Necator americanus</td>
<td>Hamster*, Nottingham, U.K.</td>
<td>Human, India</td>
<td>2006</td>
</tr>
</tbody>
</table>

* Experimental model.
of abdominal pain, dyspepsia and poor health. The second individual was a 25-year-old monk who complained of abdominal pain, diarrhoea and poor health. The complaint of poor health (OR 9.6, 95% CI: 6.4–14.5, p = 0.012) and abdominal pain (OR 6.7, 95% CI: 4.8–9.3, p = 0.024) was significantly associated with individuals infected with A. ceylanicum. No clinical complaints of statistical significance were associated with Necator infections.

4. Discussion

This study demonstrates that A. ceylanicum and A. caninum are highly endemic hookworms among stray and semi-domesticated dogs residing in temple communities in Bangkok and that zoonotic transmission of A. ceylanicum to humans is still a largely overlooked public health problem. The last detailed reports of natural infection with A. ceylanicum in humans were published over 40 years ago, between the mid 1960s to early 1970s (Anten and Zuidema, 1964; Areekul et al., 1970; Chowdhury and Schad, 1972; Velasquez and Cabrera, 1968; Yoshida et al., 1968). In Thailand, the first case report of A. ceylanicum in humans was in a 2-month-old child who died of anaemia (Suvathi et al., 1962). Areekul et al. (1970) later found 7 out of 45 (16%) hookworm positive individuals in Thailand to harbour adult worms of A. ceylanicum, often in mixed and in light infections with N. americanus. Since then, this parasite has been regarded as a ‘rare’ and ‘abnormal’ hookworm of humans (Chowdhury and Schad, 1972) and largely overlooked in human parasite surveys due to the innate difficulties associated with differentiating the two species of Ancylostoma (A. duodenale and A. ceylanicum) based on coprological examination of eggs and third stage larvae following culture (Kobayashi, 1928; Yoshida, 1971). Morphological identification of hookworm species using adult worms following expulsion chemotherapy is similarly unpleasant, time consuming and may miss mixed infections of hookworm species present in light burdens. Since the conventional methods of determining the identity of hookworms infecting humans and dogs is often labour and time consuming, this PCR-based copro-diagnostic approach offers a far more efficient method for large scaled epidemiological screening of hookworm species in dog and human populations. Microscopic identification of A. ceylanicum infection followed by PCR-based species characterisation may however have its limitations and may prove less useful for those infections that are non-patent. For example, 16 out of 183 subjects were found positive for A. ceylanicum in West Bengal (Chowdhury and Schad, 1972). Of these positive individuals, the majority harboured single sexed non-patent infections which would have been missed using microscopy, copro-culture or PCR. An immunodiagnostic method, perhaps based on faecal antigen capture ELISA may be a complementary diagnostic tool in such cases, however problems may lie in its potential cross reactivity with A. duodenale (Loukas et al., 1994, 1996). Until these diagnostic discrepancies are overcome it is likely that the prevalence of A. ceylanicum in human populations will continue to be under-reported and the significance of this important canine and feline parasitic zoonosis, remain unexplored in humans.

Poor health and abdominal pain were significantly associated with A. ceylanicum infection in this study. The epidemiological and clinical significance of A. ceylanicum however, remains largely unresolved due to the limited availability of published research data. Clinical signs, when reported, range from asymptomatic light infections to heavy infections with anaemia, complaints of lethargy and excessive hunger (Anten and Zuidema, 1964). In three separate experimental infections conducted between the late 1960s and early 1980s (Carroll and Grove, 1986; Wijers and Smit, 1966), subjects developed clinical signs similar to those described from experimental infection with A. duodenale and N. americanus in humans (Brumpt, 1952; Wright and Bickle, 2005). Further studies investigating the epidemiology, transmission dynamics and clinical significance of A. ceylanicum in a community endemic for hookworm disease will be beneficial in unravelling the true significance of this zoonosis in humans.

The low overall prevalence of hookworm infection in humans in this community could be attributed to the fact that the majority of individuals defaecated in indoor toilets and wore footwear while outdoors. Moreover, the majority of individuals cleaned canine faeces from their immediate surroundings at least once a week, preventing in some cases, the hookworm eggs from fully developing to infectivity. In this community, the importance of continued use of footwear and indoor defaecation practices should be re-enforced and encouraged along with regular checks for parasites in stool. Increased awareness and commitment from the wider community with regards to responsible pet ownership and the importance of sterilisation, vaccination and deworming programs among dogs in this community will also be of benefit in reducing the risk of transmission of zoonotic diseases.
5. Conclusion

In conclusion, this study demonstrates that humans are at risk of acquiring infection with *A. ceylanicum*, a largely forgotten canine parasitic zoonosis, in communities where this species of hookworm is endemic in dogs.

Acknowledgements

Financial support for this study was provided by the Australian Research Council and Bayer Healthcare, Animal Health, Leverkusen, Germany. Dr. Norbert Mencke of Bayer Animal Health in particular is acknowledged for his continuing support, advice and intellectual input in ensuring the success of this project. The authors would also like to thank Prof. Jerzy Behnke and Dr. Thomas Nolan from the University of Pennsylvania and Dr. Christian Epe from the University of Nottingham, Professor Gerry Mencke of Bayer Animal Health in particular is acknowledged for his continuing support, advice and intellectual input in ensuring the success of this project. The authors would also like to thank the research team at the Department of Protozoology, Mahidol University, namely Mr. Amorn Lekkla, Mr. Aongart Mahittikorn for their help in conducting fieldwork.

References


Areekul, S., Radomyos, P., Viravan, C., 1970. Preliminary report of conducting fieldwork. Lekkla, Mr. Aongart Mahittikorn for their help in

Protozoology, Mahidol University, namely Mr. Amorn hookworms for this study. The authors would also like

Pennsylvania and Dr. Christian Epe from the University

Schad and Dr. Thomas Nolan from the University of

choral attacks are largely forgotten canine parasitic zoonosis, in communities where this species of hookworm is endemic in dogs.


Setasuban, P., Vajarasthira, S., Muennoo, C., 1976. Prevalence and zoonotic potential of *Ancylostoma ceylanicum* in cats in


